Murine mammary FM3A carcinoma cells transformed with the herpes simplex virus type 1 thymidine kinase gene are highly sensitive to the growth-inhibitory properties of (E)-5-(2-bromovinyl)-2′-deoxyuridine and related compounds

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Murine mammary carcinoma (FM3A TK⁻/HSV-1 TK⁺) cells, which are thymidine kinase (TK)-deficient but have been transformed with the herpes simplex virus type 1 (HSV-1) TK gene are inhibited in their growth by (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU), (*E*)-5-(2-iodovinyl)-2'-deoxyuridine (IVDU) and (*E*)-5-(2-bromovinyl)-2'-deoxycytidine (BVDC) at 0.5, 0.5 and 0.8 ng/ml, respectively; i.e., a concentration 5000 to 20000-fold lower than that required to inhibit the growth of the corresponding wild-type FM3A/0 cells. Hence, transformation of tumor cells with the HSV-1 TK gene makes them particularly sensitive to the cytostatic action of BVDU and related compounds.

Murine mammary FM3A carcinoma *Thymidine kinase gene* *Herpes simplex virus type 1* *Cytostatic activity* (E)-*5-(2-Bromovinyl)-2′-deoxyuridine*

1. INTRODUCTION

Recently, several potent and selective antiherpetic agents have been developed, i.e. 9-(2-hydroxyethoxymethyl)guanine (acyclovir, ACV) [1], 9 - [(1,3 - dihydroxy - 2 - propoxy)methyl]guanine (DHPG) [2,3], (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) [4] and its closely related analogues (*E*)-5-(2-iodovinyl)-2′-deoxyuridine (IVDU) [4], (*E*)-5-(2-bromovinyl)-2′-deoxycytidine (BVDC) [5] and (*E*)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil (BVaraU) [6,7], 1-(2′-fluoro-2′-deoxy-1-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) [8,9], 1-(2′-fluoro-2′-deoxy-1-β-D-arabinofuranosyl)-5-methyluracil (FMAU) [9,10] and phosphonoformic acid (PFA) [11]. The selectivity of these compounds as inhibitors of herpes simplex virus (HSV) primarily depends upon a specific activation (phosphorylation) by the HSV-encoded thymidine (dThd) kinase (TK), except for PFA [12]. Indeed, upon HSV infection, a virus-specified TK is induced, which differs from human cytosol and mitochondrial TK in its physical, immunological and kinetic behavior [13]. It is endowed with deoxycytidine (dCyd) kinase activity and shows a much greater affinity for several nucleoside analogues including ACV [14], DHPG [15], BVDU [16], IVDU [16], BVDC [16], BVaraU [17], FIAC [17] and FMAU [17], than the cellular (cytosol) TK. The fact that these compounds are intensively phosphorylated by the viral TK, and only to a very limited, and often undetectable, extent by the cellular TK, apparently accounts for their low level of toxicity for the uninfected host cell.

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We have previously described a thymidylate synthetase (TS)-deficient mutant cell line (designated FM3A/TS$^-$) which was derived from mutagenized murine mammary carcinoma FM3A/0 cells [18–21]. This mutant cell line, which is auxotrophic for dThd appeared very useful to distinguish those pyrimidine nucleoside analogues that are, like dThd, incorporated into host cell DNA and stimulatory for cell growth from those nucleoside analogues that are not [20,21]. We have now constructed a TK-deficient FM3A cell line (FM3A TK$^-$) which was subsequently transformed by a fragment of HSV-1 DNA containing the gene for TK (hence designated FM3A TK$^-$/HSV-1 TK$^+$). A broad variety of both selective and non-selective antiherpetic drugs were evaluated for their inhibitory effects on the growth of FM3A/0 and FM3A TK$^-$/HSV-1 TK$^+$ cells. Those compounds whose anti-herpes activity does not depend on a specific phosphorylation by the HSV-1 TK proved equally inhibitory to the proliferation of FM3A/0 and FM3A TK$^-$/HSV-1 TK$^+$ cells. Some (but not all) of the compounds, whose inhibitory effects on HSV-1 replication depend on a specific phosphorylation by the viral TK were much more inhibitory to the growth of FM3A TK$^-$/HSV-1 TK$^+$ than FM3A/0 cells. For example, BVDU inhibited the growth of FM3A TK$^-$/HSV-1 TK$^+$ and FM3A/0 cells at 0.5 ng/ml and 11.4 μg/ml, respectively. Thus, transformation of the FM3A cells with the HSV-1 TK gene increased the cytostatic activity of BVDU 22800-fold.

2. MATERIALS AND METHODS

2.1. *Cells: growth conditions, origin and selection*

Murine FM3A cells (subclone F28-7), originally established from a spontaneous mammary carcinoma in a C3H/He mouse [22] and designated FM3A/0, were grown in 75-cm^2 tissue culture flasks (Sterilin, Teddington, England) in Eagle's MEM, supplemented with 10% (v/v) inactivated fetal calf serum (Gibco Bio-Cult, Glasgow, Scotland), 2 mM L-glutamine (Flow Laboratories, Irvine, Scotland) and 0.075% (w/v) $NaHCO_3$ (Flow Laboratories). FM3A/TS$^-$ cells were maintained in the same culture medium, supplemented with 20 μM dThd [20,21].

The FM3A TK$^-$/HSV-1 TK$^+$ cell line, which lacks host cell TK activity but contains the HSV-1 TK gene, was originally derived from a subclone of FM3A/0 cells, made deficient for host cell TK by selection in the presence of 5-bromo-2′-deoxyuridine (BDU). The HSV-1 TK gene was introduced by DNA-mediated gene transfer using pBR322 plasmid containing the 3.6-kb *Bam*HI fragment of HSV-1 DNA at a *Bam*HI site. The plasmid clone used for transformation did not contain the genes for viral DNA polymerase or ribonucleotide reductase.

From the FM3A TK$^-$/HSV-1 TK$^+$ cells a TS-deficient subclone was derived as in [22]. Shortly, FM3A TK$^-$/HSV-1 TK$^+$ cells were mutagenized with 1 μg/ml *N*-methyl-*N*′-nitro-*N*′-nitrosoguanidine for 3 h at 37°C. The cells were grown for 5 days in the presence of 10^{-5} M dThd, and then plated on agarose in the presence of 10^{-6} M 5-methyltetrahydrofolate, 2×10^{-7} M methotrexate and 10^{-5} M dThd. FM3A TK$^-$/HSV-1 TK$^+$/TS$^-$ colonies were isolated after 10 days. FM3A TK$^-$/HSV-1 TK$^+$ and FM3A TK$^-$/HSV-1 TK$^+$/TS$^-$ cells were cultured in the same medium as FM3A/0 and FM3A/TS$^-$ cells, i.e., in the absence or presence of 20 μM dThd, respectively.

2.2. *Test compounds*

The source of the test compounds was as follows: BVDU and IVDU, synthesized by R. Busson and H. Vanderhaeghe of the Rega Institute for Medical Research (Katholieke Universiteit Leuven, B-3000 Leuven), following a modification of the method described by Jones et al. [23]; BVaraU, provided by H. Machida (Yamasa Shoyu Co., Choshi, Japan), see also [24]; BVDC, provided by R.T. Walker (University of Birmingham, Birmingham, England); see also [23]; ACV, Burroughs Wellcome Co. (Research Triangle Park, NC); the 8-iodo derivative of ACV (IACV), provided by M.J. Robins (University of Alberta, Edmonton, Canada), see also [25]; FIAC and FMAU, provided by J.J. Fox (Sloan-Kettering Institute, New York, NY), see also [9,10]; DHPG, provided by J.P.H. Verheyden (Syntex Research, Palo Alto, CA); 5-(2-chloroethyl)-2′-deoxyuridine (CEDU), provided by B. Rosenwirth (Sandoz Forschungsinstitut, Vienna, Austria), see also [26]; 1-β-D-arabinofuranosylthymine (araT), provided by H. Machida (Yamasa Shoyu Co., Choshi, Japan); 5-fluoro-2′-deoxyuridine (FDU), Aldrich (Milwaukee, WI); BDU, Sigma Chemical Com-

pany (St. Louis, MO); 5-iodo-2′-deoxyuridine (IDU), Sigma; 5-trifluoromethyl-2′-deoxyuridine (TFT), P-L Biochemicals (Milwaukee, WI); 5-ethyl-2′-deoxyuridine (EDU), Robugen GmbH (Esslingen, FRG), see also [27,28]; 5-propyl-2′-deoxyuridine (PDU), [29]; 9-β-D-arabinofuranosyladenine (araA), Sigma; 1-β-D-arabinofuranosylcytosine (araC), Upjohn Company (Puurs, Belgium); and PFA, provided by B. Öberg (Astra Läkemedel AB, Södertälje, Sweden). The therapeutic potentials of these anti-herpes agents have been reviewed in [30,31].

2.3. *Inhibition of tumor cell growth*

All assays were performed in 96-multiwell microtest plates (Falcon, Becton Dickinson, Oxnard, CA). To each well were added 5×10^4 cells and varying amounts of the test compounds. The cells were then allowed to proliferate at 37°C in a humidified, CO_2-controlled atmosphere. In preliminary assays it was assessed that the growth of the cells was linear for up to 72 h. In the cell growth-inhibition experiments, the incubation was stopped at 48 h. The cells were then enumerated in a Coulter Counter (Coulter Electronics, Harpenden, England). The cell growth-inhibitory effects of the test compounds are expressed in ID_{50}, or the inhibitory dose required to reduce the final cell number by 50%.

3. RESULTS AND DISCUSSION

3.1. *Inhibitory effects of anti-herpes agents on the proliferation of FM3A/0 and FM3A TK⁻/HSV-1 TK⁺ cells*

A wide series of anti-herpes agents were evaluated for their inhibitory effects on the growth of FM3A/0 and FM3A TK⁻/HSV-1 TK⁺ cells (table 1). Based on whether the compounds act as preferential substrates for the HSV-1 TK [12], distinction was made between selective and non-selective anti-herpesvirus agents (although it should be recognized that some of the compounds belonging to the latter category, i.e., araA and PFA, may interact specifically with the viral DNA polymerase).

Among those compounds whose anti-herpes activity depends on a specific phosphorylation by the viral TK, several congeners were much more inhibitory to the proliferation of FM3A TK⁻/HSV-1 TK⁺ than of FM3A/0 cells; i.e., BVDU (22800-fold), BVDC (20250-fold), IVDU (5600-fold), araT (678-fold) and DHPG (130-fold). Clearly, phosphorylation of these compounds by the viral TK is required for their inhibitory effect on the growth of HSV-1 transformed cells. Although obligatory, this phosphorylation may not be sufficient for a cytostatic effect of the anti-herpes agents on HSV-1 transformed cells, since several other compounds, ACV, FMAU, EDU and BVaraU, which also

Table 1

Inhibitory effects of anti-herpes agents on the proliferation of FM3A/0 and FM3A TK⁻/HSV-1 TK⁺ cells

Compound[a]	ID_{50} (μg/ml)		ID_{50} (FM3A/0)
	FM3A/0	FM3A TK⁻/HSV-1 TK⁺	ID_{50} (FM3A TK⁻/HSV-1 TK⁺)
Selective anti-herpes agents			
BVDU	11.4	0.0005	22800
BVDC	16.2	0.0008	20250
IVDU	2.80	0.0005	5600
araT	238	0.351	678
DHPG	37.5	0.288	130
FMAU	5.92	1.20	4.9
ACV	27.2	7.32	3.7
IACV	180	74	2.4
FIAC	>100	>10[b]	–
CEDU	35.4	>10[b]	–
EDU	9.47	>10[b]	–
BVaraU	>300	>300	–
PDU	>1000	>1000	–
Non-selective anti-herpes agents			
araA	15.4	3.72	4.1
TFT	0.007	0.004	1.7
PFA	221	137	1.6
araC	0.211	0.150	1.4
FDU	0.0005	0.0006	0.8
BDU	38.5	620	0.06
IDU	7.38	454	0.02

[a] Considered as 'selective' if preferentially phosphorylated by the HSV-1 TK [12]

[b] ID_{50} value could not be measured accurately within the concentration range of 10–1000 μg/ml

depend on a specific phosphorylation by the viral TK, were found not to inhibit FM3A TK^-/HSV-1 TK^+ cell growth to a significantly greater extent than FM3A/0 cell growth. The contrast between BVDU and BVaraU is particularly striking. Both compounds are equally inhibitory to HSV-1 replication in human fibroblast cell cultures [6]. Nevertheless, BVDU is inhibitory to the replication of HSV-1-transformed FM3A cells at a concentration of 0.5 ng/ml, whereas BVaraU does not affect the growth of these cells even at 300 μg/ml.

It is not yet clear why there are such dramatic differences in the cytostatic potencies of the selective anti-HSV-1 agents for HSV-1-transformed cells. All these agents inhibit an acute HSV-1 infection within the concentration range of 0.5–0.005 μg/ml [1–10]. Yet, their ID_{50} for HSV-1-transformed cells varies over more than a 10^6-fold range. The relatively poor activity of ACV, IACV, FMAU and FIAC, and inactivity of BVaraU and PDU, as inhibitors of HSV-1-transformed cell growth may be related to several factors; i.e., these compounds may not be adequately phosphorylated within the transformed cells, or, if they are, they may fail to reach the target(s) for a cytostatic action.

It is tempting to correlate the cytostatic effects of BVDU, BVDC and IVDU on HSV-1-transformed cells to their incorporation into the host DNA, and, conversely, to attribute the inactivity of BVaraU to a lack of incorporation into cell DNA. Analogues of 1-β-D-arabinofuranosyluracil (araU) are, in general, very poor substrates for DNA polymerase, and do not act as an alternate substrate for DNA elongation in the absence of dTTP. This has been shown directly for a series of 5-substituted araUTP analogues [32] as well as BVaraUTP [7,33]. Thus, the incorporation of the nucleoside analogues into host cell DNA may be a critical determinant in their cytostatic action. Whether the compound is incorporated within the interior of the DNA chain or at the 3′-terminal is another important consideration. Compounds like BVDU, FIAC and DHPG can be incorporated via an internucleotide linkage [12,13,34], and, consequently, their incorporation may be irreversible. In contrast, ACV and BVaraU are incorporated only at the 3′-terminal [7,13,35], and such an incorporation may be reversible, since endonucleases could remove terminal oligonucleotides containing ACV or BVaraU. It would now seem mandatory to investigate to what extent the different compounds are processed, phosphorylated, metabolized and incorporated into DNA of HSV-1-transformed cells.

As expected, those compounds whose potent anti-herpes activity does not depend on a specific phosphorylation by the HSV-1 TK proved equally cytostatic for FM3A/0 and FM3A TK^-/HSV-1 TK^+ cells (table 1). In fact, BDU and IDU were even more inhibitory to the proliferation of FM3A/0 than of FM3A TK^-/HSV-1 TK^+ cells. Perhaps a decreased phosphorylation of these compounds by the FM3A TK^-/HSV-1 TK^+ cells may account for the decreased cytostatic activity, since FM3A TK^-/HSV-1 TK^+ cells possess only 35.6% of the TK activity expressed in FM3A/0 cells (in preparation).

3.2. *Stimulatory effects of anti-herpes agents on the proliferation of FM3A/TS^- and FM3A TK^-/HSV-1 TK^+/TS^- cells*

Based on the premise [20,21] that stimulatory effects of dThd analogues on the growth of dThd-auxotrophic FM3A/TS^- cells may be considered as indicative for their incorporation into host cell DNA, all the compounds listed in table 1 were examined for their capacity to substitute for dThd in sustaining the growth of FM3A TK^-/HSV-1 TK^+/TS^- cells. Only BDU and IDU were able to do so (at ~10 μg/ml; not shown). BVDU, BVDC, IVDU, araT and DHPG were unable to sustain the growth of FM3A TK^-/HSV-1 TK^+/TS^- cells. Apparently, their potential to stimulate the growth of these TS^- cells was counteracted by their cytostatic action, as already evident from their inhibitory effect on the growth of FM3A TK^-/HSV TK^+ cells (table 1). ACV, IACV and BVaraU were also unable to sustain growth of the FM3A TK^-/HSV-1 TK^+/TS^- cells, probably because they act as DNA chain terminators. FDU, TFT and PDU, which have been shown to be unable to sustain the growth of FM3A/TS^- cell growth [21], did not sustain the growth of FM3A TK^-/HSV-1 TK^+/TS^- cells either.

4. CONCLUSION

The major observation emerging from these studies is the highly potent and selective cytostatic

effect of BVDU, BVDC and IVDU, at concentrations as low as 0.5–0.8 ng/ml, on murine mammary carcinoma cells transformed with the HSV-1 TK gene. While the exact mechanism by which BVDU achieves this inhibitory activity is subject to further study, the fact that transformation of tumor cells with the HSV-1 TK gene makes them exquisitely sensitive to the cytostatic action of such selective anti-herpes drugs as BVDU may have far-reaching implications, both fundamental and therapeutic.

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